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(54) Title: OXIDOREDUCTASE ACTIVITY OF MANGANIC PORPHYRINS

(57) Abstract

The present invention relates, in general, to porphyrin complexes and, in particular, to methods of using porphyrin complexes to impart oxidoreductase activity.

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OXIDOREDUCTASE ACTIVITY OF MANGANIC PORPHYRINS

TECHNICAL FIELD

The present invention relates, in general, to porphyrin complexes and, in particular, to methods of using porphyrin complexes to impart oxidoreductase activity.

BACKGROUND

Superoxide radical (O_2^-), generated during both spontaneous and enzyme-catalyzed oxidations, is catalytically scavenged by superoxide dismutases (SODs); which, by so doing, provide an important defense (Beyer et al, Prog. Nucl. Acids Res. 40:221 (1991), Fridovich, J. Biol. Chem. 264:7761 (1989)). O_2^-, if not removed by SOD, can: initiate free radical chain oxidations of low molecular weight reductants (Nishikimi, Arch. Biochem. Biophys. 166:273 (1975), Ballou et al, Biochem. Biophys. Res. Commun. 36:898 (1969), Fridovich et al, J. Biol. Chem. 233:1578 (1958), McCord et al, J. Biol. Chem. 243:5753 (1968), McCord et al, J. Biol. Chem. 244:6056 (1969), McCord et al, J. Biol. Chem. 244:6049 (1969)); inactivate enzymes (Kuo et al, J. Biol. Chem. 262:4724 (1987), Takabatake et al, Chem. Pharm. Bull. 40:1644 (1992), Smyk-Randall et al, Free Rad. Biol. Med. 14:609 (1993), Gardner et al, J. Biol. Chem. 266:1478 (1991), Gardner et al, J. Biol. Chem. 266:1478 (1991), Gardner et al, J. Biol.

Chem. 266:19328 (1991), Flint et al, J. Biol. Chem. 268:22369 (1993)); and can give rise to very reactive hydroxyl or alkoxyl radicals through iron- or copper-, catalyzed interactions with HOOH or ROOH (Sutton et al, Free Rad. Biol. Med. 6:53 (1989), Smith et al, Free Rad. Res. Commun. 8:101 (1990), Nakae et al, Arch. Biochem. Biophys. 279:315 (1990), Tushelasvili et al, J. Biol. Chem. 266:6401 (1991), Mello-Filho et al, Mutat. Res. 251:109 (1991), Halliwell et al, FEBS Lett. 307:109 (1992)). O_2^- can also react, at a diffusion-limited rate, with NO; yielding peroxynitrite (Huie et al, Free Rad. Res. Commun. 18:195 (1993)).

It is reasonable, therefore, that O_2^- should be a participant in a variety of physiological and pathological processes. The ability of SOD to ameliorate reperfusion injury (Concannon et al, Microsurgery 12:18 (1991), Triana et al, Circ. Res. 69:731 (1991), Erlansson et al, Free Rad. Biol. Med. 9:59 (1990), Fujita et al, Biochem. Biophys. Res. Commun. 129:191 (1992), Hatori et al, Free Rad. Biol. Med. 13:137)), inflammations (Ward et al, Free Rad. Biol. Med. 5:403 (1988), Parizada et al, Free Rad. Res. Commun. 15:297 (1991), Oyanagui et al, Biochem. Pharmacol. 42:991 (1991)), multiorgan failure (Marzi et al, J. Trauma 35:110 (1993)), brain trauma (Muizeloar, Ann. Emerg. Med. 22:1014 (1993)), and other conditions (Flohé, Mol. Cell. Biochem. 84:123 (1988), Gorecki et al, Free Rad. Res. Commun. 12-13:401-410 (1991)), indicates that this is the case. It is apparent therefore that mimics of SOD activity are useful

pharmaceutical agents. The ideal mimic should be active, stable, and specific for its substrate (O_2^-). A number of metal complexes have been reported to catalyze the dismutation of O_2^- (see, for example, McLaughlin et al, Inorg. Chem. 32:941 (1993), Tian et al, Biochem. Biophys. Res. Commun. 191:646 (1993), Kitajima et al, Inorg. Chem. 32:1879 (1993), Baudry et al, Biochem. Biophys. Res. Commun. 192:964 (1993)). Even the best of the compounds described to date, however, exhibit only ~1% of the activity of SOD, are not stable to EDTA, and/or can catalyze reactions in addition to the dismutation of O_2^-.

SUMMARY OF THE INVENTION

The present invention relates to a method of effecting oxidoreduction in a sample containing O_2^-. The method comprises contacting the sample with a metallic porphyrin complex having substituents on the methine carbons under conditions such that the complex is reduced and reoxidized by the O_2^-.

Objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Mn porphyrins. Shown are the porphyrin structures, names, and abbreviations used herein.

Figure 2. Reduction of cytochrome c^{3+} in the presence of MnTMPyP. Shown are the absorbance vs. time traces of cyt c^{3+} reduction in the cytochrome c^{3+} assay. Trace A is the reduction of cyt c^{3+} in the absence of MnTMPyP. Trace B) 0.15 μM MnTMPyP, C) 0.75 μM MnTMPyP, and D) 1.15 μM MnTMPyP. Conditions are as follows: 8.9 μM cyt c^{3}, 35 μM xanthine, 3.5 μL of 1 μM xanthine oxidase, 0.05 M phosphate buffer, 0.10 mM EDTA, pH 7.80, 21°C.

Figure 3. Asada plot of MnTMPyP SOD activity. The line through the data is the best fit linear regression line (r^2 = 0.98). Vo = $\Delta AU_{550\ nm}$/min for cyt c^{3} in the absence of MnTMPyP, and Vi = $\Delta AU_{550\ nm}$/min for c^{3+} reduction in the presence of various concentrations of MnTMPyP, as shown on the x-axis. Conditions are as follows: 8.9 μM cyt c^{3+}, 35 μM xanthine, 3.5 μL of 1 μM xanthine oxidase, 0.05 M phosphate buffer, 0.10 mM EDTA, pH 7.80, 21°C.

Figure 4. Growth induction of sodAsodB *E. coli* mutant with MnTMPyP and MnTBAP. This figure shows the growth curves of *E. coli* AB1157, and J1I132 in the presence and absence of MnTMPyP and MnTBAP. The traces are as follows: 1. A) AB1157, B, C, D) JI132 in the presence of 21, 51, 100 μM MnTMPyP, respectively, and E) JI132. 2. A') AB1157, B', C', D') JI132 in the presence of 37, 54, 86 μM MnTBAP, respectively, and E') JI132. Conditions are as follows: M9 salts, phosphate

buffer in tap water, 2% casamino acids, 0.2% glucose, 37°C.

Figure 5. Growth induction of sodAsodB *E. coli* with MnTMPyP in minimal media. This figure shows the growth curves in minimal media of *E. coli* strain AB1157 (black squares) and JI132 (open triangles) in the presence and absence of 24 μM MnTMPyP (dotted lines). Traces are as follows: A) AB1157 + 24 μM MnTMPyP, B) AB1157 alone, C) JI132 + 24 μM MnTMPyP, D) JI132 alone. Conditions are as follows: M9 salts in tap water, 0.2% glucose, 0.50 mM each of threonine, leucine, proline, histidine, and arginine, grown at 37°C.

Figure 6. Protection of *E. coli* by MnTMPyP against paraquat induced toxicity. This Figure shows the growth curve of the AB1157 strain of *E. coli* in rich media. Traces are of AB1157 A) alone, B) +20 μM MnTMPyP, C) +10 μM paraquat and 20 μM MnTMPyP, and D) +10 μM paraquat. Conditions are as follows: M9 salts in tap water, 2% casamino acids, 0.2% glucose, 37°C.

Figure 7. Effect of MnTMPyP on the oxidation of NADPH by cell extract. Reactions were performed with 0.1 mM NADPH in 50 mM potassium phosphate in 3.0 ml at 25°C and pH 7.3 and absorbance changes were recorded at 340 nM. Expt. 1 - Cell extract (CE) was added to 32 μg protein/ml at the first arrow; MnTMPyP was added to

12.5 μM at the second arrow; and Cu,ZnSOD was added to 50 μg/ml at the third arrow. Expt. 2 - MnTMPyP to 25 μM and then cell extract to 32 μg protein/ml -- as indicated. Numbers over the lines give the slopes in terms of ΔA_{340}nm/min.

Figure 8 shows the effect of MnTMPyP on the oxidation of NADPH by thioredoxin reductase. Reaction conditions as in the legend of Figure 7 except that thioredoxin reductase (TR) was used at 4 μg/ml in place of cell extract. In expt. 1, paraquat (PQ), where indicated, was added to 4.0 mM, in experiment 2, MnTMPyP was added to 25 μM, and, in expt. 3, the metal-free porphyrin was used in place of MnTMPyP.

Figure 9. LB medium blunts the ability of MnTMPyP to stimulate NADPH oxidation by thioredoxin reductase. Reaction mixtures of 3.0 ml contained 0.1 mM NADPH and additional components in 50 mM potassium phosphate buffer at pH 7.3 and at 25°C. Expt. 1 - 25 μM MnTMPyP present at outset and thioredoxin reductase (TR) added to 2.0 μg/ml at the arrow. Expt. 2 - as in Expt. 1 except that 20% of the phosphate buffer was replaced by LB medium. Expt. 3 - 4.0 mM PQ present at the outset and TR added to 0.67 μg/ml at the arrow. Expt. 4 - as in expt. 3 except that 20% of the phosphate buffer was replaced by LB medium.

Figure 10. Oxidation of GSH catalyzed by MnTMPyP. Reaction mixtures contained 10 mM GSH in 50 mM buffer

at the arrows. MnTMPyP was added to 25 μM or to an additional 50 μM, as indicated and O_2 uptake was monitored. Expt. 1 in phosphate at pH 7.3 and expt. 2 in Tris-HCl at pH 7.8.

Figure 11. Optical absorption spectra of MnTMPyP. The MnTMPyP was at 25 μM in 50 mM Tris-HCl at pH 7.8 and at 25°C. Spectrum 1 - anaerobic; spectrum 2 - anaerobic plus 5 mM GSH, recorded 5 minutes after mixing.

DETAILED DESCRIPTION OF THE INVENTION

Manganese porphyrins are well characterized and stable (Collman et al, Science 261:1404 (1993), Tsang et al, Inorg. Chem. 29:2848 (1990), Harriman et al, J. Chem. Soc. Faraday Trans. 275:1532 (1979)). Moreover, such compounds have been reported to catalyze the dismutation of O_2^- with rate constants as high as 4×10^7 M^{-1} s^{-1} (Farragi, M. in Oxygen Radicals in Chemistry and Biology (Bors, W., Saran, M., and Tait, D., eds.) pp. 419-430, Walter de Gruyter, Publ., Berlin (1984), Pasternak et al, J. Inorg. Biochem. 15:261 (1981)). The present invention results from the discovery that manganic porphyrins exhibit reversible redox behavoir. More specifically, the invention results from the finding that Mn(III) 5, 10, 15, 20-tetrakis(1-methyl-4-pyridyl)-21H,23H-porphine tetra-p-tosylate (MnTMPyP) *in vivo* is reduced enzymatically at the expense of NADPH and non-enzymatically by GSH, with

a rate constant for reoxidation of the reduced form by O_2^- of 4 x 10^9 $M^{-1}s^{-1}$. The compound thus acts as a NADPH/GSH:O_2^- oxidoreductase *in vivo*. Such action requires replenishment of NADPH and GSH; that can be achieved at the expense of carbon source (eg glucose).

MnTMPyP is not the only manganic porphyrin that imparts an NADPH:O_2^- oxioreductase activity and acts like a GSH:O_2^- oxidoreductase. Several manganic porphyrins, with substituents on the methine carbons, were prepared and examined for stability, redox behavior, catalysis of the dismutation of superoxide radical (O_2^-), and for the ability to protect a SOD-null strain of *E. coli* against dissolved oxygen, and a SOD-competent strain against paraquat (see Example that follows). All of the compounds tested exhibited reversible redox behavoir and were stable to EDTA in both the oxidized and reduced states, and several were able to catalyze the dismutation of O_2^- with rate constants of ~10^7 $M^{-1}s^{-1}$. The marked protective effects of certain of these compounds exceeded that which could be anticipated on the basis of such rate constants.

While the present invention is described with reference to manganic porphyrins bearing substituents on the methine bridge carbons, other metals can also be used. Examples of such metals include iron (Fe(III)→Fe(II)), cobalt (Co(III)→Co(II)), nickle (Ni(II)→Ni(I)) and copper (Cu(II)→Cu(I)). The complexes of the present invention can be obtained

commercially or can be prepared using methods known in the art (see also Example that follows).

The oxidoreductase function of the molecules (complexes) of the invention permits their administration as pharmaceuticals in concentrations sufficiently low to avoid problems associated with toxicity, which concentrations are sufficiently high to achieve the effect sought. Appropriate doses can be readily determined by one skilled in the art, given the present disclosure (as an example, a dose of 1-50 mg/kg can be used). As regards the formulation of the molecules of the invention as compositions and as regards methods of using the molecules of the invention (eg in autoxidation inhibition and in protection), see Application No. 08/089,813 (the entire contents of which is incorporated herein by reference) (see also USP 5,223,538 and USP 5,227,405).

The invention is illustrated by way of reference to the non-limiting Example that follows.

Example

Porphyrin Mimetics Substitute for SOD *In Vivo*

Preparation of Mn Porphyrins - Complexes of H_2-TMPyP and H_2-TMAP, with Mn, Fe or Co, were prepared by the method of Pasternack et al, Biochemistry 22:2406 (1983). Metal ligation was followed spectrophotometrically. The MnTMPyP complex was characterized in terms of its Soret band at 463 nm,

E_m = 9.2 x 10^4 M^{-1} cm^{-1} (Harriman et al, J. Chem. Soc. Faraday Trans. 275:1532 (1979); Pasternack et al, Biochemistry 22:2406 (1983)). The Soret band for the MnTMAP complex was at 465 nm with E_m = 9.2 x 10^4 M^{-1} cm^{-1}. As judged by constancy of their absorption spectra, these compounds were stable from pH 1-9. Moreover, the Mn-porphyrins did not oligomerize in the range 0-3 μM, since there were no deviations from Beers law. The Mn complexes of H_2-TPyP and of H_2-TBAP were prepared by the method of Harriman and Porter (J. Chem. Soc. Faraday Trans. 275:1532 (1979)). For MnTPyP the Sorêt band was at 463 nm and E was 9 x 10^4 M^{-1} cm^{-1}; while for MnTBAP the corresponding values were 468 nm and 9.3 x 10^4 M^{-1} cm^{-1} (Harriman and Porter, J. Chem. Soc. Faraday Trans. 275:1532 (1979)).

Assays - Catalysis of the dismutation of O_2^- was measured by using xanthine oxidase plus xanthine as the source of O_2^- and ferricytochrome c as the indicating scavenger of O_2^- (McCord et al, J. Biol. Chem. 244:6049 (1969)). Rates of reduction of cytochrome c were followed at 550 nm with a Beckman model DU-70. Assays were conducted in the absence and in the presence of 0.1 mM EDTA in 50 mM potassium phosphate, pH 7.8 and at 25°C. Rate constants for reaction of the metal porphyrins with O_2^- was based on their competition with cytochrome c for reaction with O_2^-, using $k_{O2^-, \text{cyt c}}$ = 3.0 x 10^6 $M^{-1}s^{-1}$ (Koppenol et al, Israel J. Chem. 24:11 (1984)). Possible interference by inhibition of the xanthine oxidase reaction by the test compounds was

examined by following the rate of accumulation of urate at 295 nm in the absence of cytochrome c. The oxidation of NADPH was followed at 340 nm. Anaerobic measurements were made using an anaerobic cuvette (Hodgson et al, Anal. Biochem. 51:470 (1973)) purged with N_2 which had been passed over a bed of hot copper foil to remove traces of O_2 and which was conducted to the cuvette through copper tubing. O_2 uptake was followed polarographically with a Clark electrode. All measurements were at 25°C.

Culture of E. coli - Rates of growth of cultures of *E. coli* were followed turbidimetrically at 700 nm to minimize interferences from the absorbance of test compounds. Culture media were prepared as described by Maniatis et al, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press Cold Spring Harbor, NY. Supplemented medium contained 0.2% glucose, 0.2% casamino acids and M9 salts in tap water, pH adjusted to 7.0. Minimal medium contained 0.2% glucose and 0.5 mM each of leucine, threonine, proline, arginine and histidine, plus M9 salts in tap water, pH adjusted to 7.0. In some cases Mn-poor medium was prepared by substituting MOPS for the phosphate present in M9 and by using Chelex-100 to remove contaminating metal cations. Cu(II), Zn(II) and Fe(II) were then added to ~1 mg/L. All media were further supplemented with 30 mg/L of thiamine and of D-pantothenic acid. When added to media, test compounds were filter-sterilized. Extracts of cells grown in M-9 medium supplemented with

0.2% casamino acids were prepared, after washing with 50 mM sodium phosphate at pH 7.3 and resuspension in this buffer, by use of the French Press. The lysate was clarified by centrifugation. LB adjusted to pH 7.0 medium was prepared as described (Maniatis et al, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press Cold Spring Harbor, NY). Protein was measured by the method of Lowry et al (J. Biol. Chem. 193:265 (1951)).

Electrochemical Characterization of Test Compounds - Cyclic voltammetry was performed using a PAR Model 175 potentiostat and a PAR Model 173 programmer. The working electrode was a polished Pt button electrode pretreated by sonication in 1 N HCl. An Ag/AgCl electrode (Bioanalytical Systems) served as the reference (-200 mV vs NHE), and a 1 mm diameter Pt wire auxiliary electrode. Cyclic voltammetry was performed in 0.05 M phosphate buffer, pH 7.8, 0.1 M NaCl, 21°C. $E_{½}$ values were obtained by taking the average of the E_{pc} and $E_{p}a$ values. Typical scan rates were from 100 to 500 mV/sec. The electrochemistry was performed in Argon purged solution with added $Na_2S_2O_4$ to scavenge trace O_2. The added $Na_2S_2O_4$ had no influence on the $E_{½}$ values obtained for the Mn porphyrins, but increased the current response at the electrode surface by reducing the competition of Mn(II) porphyrin with electrochemically reduced O_2 species. 1-Methyl imidazole was also present in the solution to compete for the axial coordination sites on the Mn porphyrins.

The 1-methyl imidazole had no influence on the $E_{1/2}$ values obtained, but decreased the ΔE_{pp} values (E_pa-E_{pc}). When 1-methyl imidazole was added, the pH of the solution was adjusted to pH 7.8 using 1 N HCl.

Stability of Mn Porphyrins - The structures, proper names and abbreviated designations for the test compounds are given in Figure 1. The optical spectra of MnTPyP, MnTMPyP, MnTMAP, MnTBAP and MnTMINP were independent of pH in the range 1.0-9.0. These complexes were unaffected by 0.10 mM EDTA; which represented at least a ten-fold excess of EDTA over the test compounds. At pH 7.8 these complexes could be reduced with dithionite and then reoxidized by O_2 without loss and this could be done in the presence of excess EDTA. It follows that both the Mn(II) and the Mn(III) forms of these complexes were stable towards EDTA.

Activity in vitro - MnTMPyP exhibited SOD-like activity in the xanthine oxidase-cytochrome c assay, in the presence of a ~100-fold molar excess of EDTA (see Figure 2). MnTMPyP, tested up to 6 μM, did not inhibit the xanthine oxidase reaction and the optical spectrum of this porphyrin was not changed by exposure to this reaction under the conditions of the assay for SOD activity (McCord et al, J. Biol. Chem. 244:6049 (1969)) but with cytochrome c omitted. These results are in accord with the conclusions of Faraggi (Oxygen Radicals in Chemistry and Biology (Bors, W., Saran, M., and

Tait, D. Eds) pp. 419-430, Walter de Gruyter, Publ., Berlin (1984)). Fe(III) TMPyP has previously been noted to react with H_2O_2 (Pasternack et al, J. Am. Chem. Soc. 101:1026 (1979)). The Sorêt band of the Mn(III)TMPyP was similarly diminished by H_2O_2 at 1.0 or 10.0 mM. However, when [H_2O_2] was only 0.1 mM the bleaching of the Sorêt band of 12.5 μM Mn(III)TMPyP was very slow; being less than 1% loss of absorbance per minute at 25°C. It follows that attack by H_2O_2 would be insignificant in catalase-proficient cells.

Inhibition of cytochrome c reduction by MnTMPyP, when plotted according to Sawada and Yamazaki (Biochem. Biophys. Acta 327:257 (1973)), yielded a straight line (Figure 3) from which IC_{50} was found to be 0.7 ($\pm$0.07) μM and from this the rate constant for the reaction of O_2^- with MnTMPyP was found to be 3.9×10^7 $M^{-1}s^{-1}$ at pH 7.8. This agrees very well with the rate constant of 4.0×10^7 $M^{-1}s^{-1}$ determined by Faraggi (Oxygen Radicals in Chemistry and Biology (Bors, W., Saran, M., and Tait, D. Eds) pp. 419-430, Walter de Gruyter, Publ., Berlin (1984)), at pH 8.0, through pulse radiolysis. It appears that Mn(III)TMPyP acts as a SOD-mimic, under the conditions of the SOD assay, without causing detectable side reactions. Comparable data for all of the Mn-porphyrins examined is presented in Table I. It should also be noted that MnTMPyP was apparently unaffected by the H_2O_2 which accumulates during the xanthine oxidase reaction since its catalytic activity was not affected by 450 units/ml of catalase.

TABLE I

Results of the Cyclic Voltammetry and the Cytochrome c Assays of the Mn Porphyrins

Mn Porphyrin	Charge (pH 7.8)	$E_{1/2}$ (mV, NHE)[a]	IC_{50} (μM)[b]	k_{cat} ($M^{-1}s^{-1}$)[b]
MnTMPyP	+5	+60	0.70	3.9×10^7
MnTPyP	+1	0	c	c
MnTMAP	+5	-100	21	1.6×10^6
MnTMINP	+5	+44	c	c
MnTBAP	-3	+110	d	d

a) Conditions: 0.05 M phosphate buffer, 0.10 M NaCl pH 7.7-7.8, 0.4 to 1.0 mM Mn porphyrin, 0.5-1 equivalent of $Na_2S_2O_4$, 2 mM 1-methyl imidazole. Reproducibility of $E_{1/2}$ = ±10 mV.

b) Conditions: 0.05 M phosphate buffer, 0.10 mM EDTA, pH 7.8, 40 μM xanthine, ca 10 μM cyt c^{3+}. Reproducibility of IC_{50} = ±10%.

c) These Mn porphyrins were not active in our assay conditions.

d) MnTBAP interfered with the production of urate, in the presence and absence of 0.1 mM EDTA.

Electrochemical Behavior - Cyclic voltammetry indicated reversible redox behavior for all of the Mn-porphyrins examined; as evidenced by $\Delta E_{pp} \leq 100$ mV at scan rates in the range 100-500 mV/sec. Moreover, in the absence of O_2, peak currents for the cathodic and anodic waves were equivalent, also indicative of reversibility. The results of cyclic voltammetry are given in Table 1. $E_{1/2}$ for MnTMPyP is in agreement with Faraggi (Oxygen Radicals in Chemistry and Biology (Bors, W., Saran, M., and Tait, D. Eds) pp. 419-430, Walter de Gruyter, Publ., Berlin (1984)); whereas the value for MnTMAP obtained is 30 mV negative of the previously reported value. Sodium dithionite, which was used to remove traces of O_2, and thus to eliminate interferences from the cathodic reduction of O_2, did not itself interfere in the potential region used during these measurements.

Activity in vivo - SOD-null *E. coli* exhibit several dioxygen-dependent auxotrophies and grow slowly aerobically, whereas they grow as well as the SOD-competent strain under anaerobic conditions (Carlioz et al, EMBO J. 5:623 (1986); Imlay et al, J. Bacteriol. 174:953 (1992)). As shown in Figure 4, either MnTMPyP or MnTBAP increased the growth of the SOD-null JI132 strain (Imlay et al, J. Bacteriol. 169:2967 (1987)) in a glucose-salts medium supplemented with 0.2% casamino acids. In a simpler medium, the SOD-null grew very slowly under aerobic conditions and, as shown in Figure 5, MnTMPyP markedly increased this rate of growth. The protection effect of MnTMPyP was also seen

when the SOD-null was grown in a manganese-deficient medium buffered with MOPS in place of phosphate.

Several Mn-porphyrins were compared for their abilities to catalyze the dismutation of O_2^- (Table I) and to increase the aerobic growth of the SOD-null strain (Table II). Their order of SOD-like activities *in vitro* was MnTMPyP>MnTMAP>MnTPyP=MnTMINP=0. In general the SOD-like activities of these complexes assayed *in vitro* paralleled their activity *in vivo* with the exception of MnTPyP which was active *in vivo* although inactive *in vitro*. FeTMPyP, which was active as a SOD-mimic (Oxygen Radicals in Chemistry and Biology (Bors, W., Saran, M., and Tait, D. Eds) pp. 419-430, Walter de Gruyter, Publ., Berlin (1984)), was toxic at 20 μM and inhibited the growth of SOD-competent *E. coli*. The metal-free porphyrins caused a slight growth inhibition, as did EDTA, probably by diminishing the availability of essential trace metals.

TABLE II

Doubling Times for E. coli Strains AB1157 and JI132 in the Presence of Mn Porphyrins

		None	CoTMPyP	MnTMPyP	MnTPyP	MnTMAP	MnTBAP
		t_D (min)[a], [concentration of Mn porphyrin] (μM)					
2% supplement of casamino acids[b]	AB1157	48	-	48 [50]	-	-	48 [50]
	JI132	240	240 [27]	60 [25]	90 [19]	210 [55]	120 [37]
5 essential amino acids [c]	AB1157	130	130 [23]	130 [50]	-	-	-
	JI132	780	780 [23]	240 [24]	540 [10]	-	-

a) t_D was calculated as the time it takes for the optical density at 700 nm of the sample to double during the log-phase growth period. With the exception of the CoTMPyP growth studies, these results are the average of at least two trials.

b) Conditions: M9 salts in phosphate buffer, tap water, 0.2% glucose, 2% casamino acids, shaken at 37°C.

c) Conditions: M9 salts in phosphate buffer, tap water, 0.2% glucose, 0.50 mM each of threonine, leucine, proline, histidine, and arginine, 37°C.

Mn(II), per se, at 2 or 95 μM, did not perceptibly overcome the growth-inhibiting effect of 30 μM paraquat. In contrast, 20 μM MnTMPyP eliminated, virtually completely, the growth inhibition imposed upon AB1157 (Imlay et al, J. Bacteriol. 174:953 (1992)) by 10 μM paraquat (Figure 6). Furthermore, 25 μM MnTMPyP was found to eliminate the growth inhibitory effect of 20 μM paraquat and to partially alleviate the growth inhibition caused by 40 μM paraquat on the SOD-competent strain. MnTPyP was similarly protective, but to a lesser degree. MnTMPyP was also able to overcome the effect of 5 μM paraquat on the SOD-null strain. The protective effects of MnTMPyP, so apparent in the amino acid-supplemented glucose plus salts medium, could not be seen in LB medium. This was due to inactivation of the MnTMPyP by a component of the LB medium.

Reduction of MnTMPyP - Centrifugation of *E. coli* (JI132 or AB1157) which had been grown in LB containing 25 μM MnTMPyP revealed that the cell pellet was red. Since this is the color of the Mn(III) complex, it establishes that the complex is taken into the cells. In contrast, cells grown in the glucose plus salts medium, supplemented with 0.2% casamino acids, were green; while the suspending medium was red. Dithionite converted the Mn(III) TMPyP to a green product, presumably the Mn(II) complex. It thus appears that Mn(III) TMPyP becomes reduced within *E. coli* and that

some component of LB medium converts it to a form which cannot be thus reduced.

Anaerobic incubation of 25 μM TMPyP with cell extract plus 0.1 mM NADPH, in phosphate at pH 7.3 or in Tris at pH 7.8, produced the green color and subsequent aeration restored the original red color. It follows that *E. coli* extract contains an NADPH:Mn(III) TMPyP oxidoreductase. Given that the Mn(II) porphyrin complexes can autoxidize (Oxygen Radicals in Chemistry and Biology (Bors, W., Saran, M., and Tait, D. Eds) pp. 419-430, Walter de Gruyter, Publ., Berlin (1984)), it would be expected that Mn(III)TMPyP should catalytically increase the oxidation of NADPH by extracts of *E. coli*. Trace 1 in Figure 7 demonstrates that this was the case and that SOD had no effect on this oxidation of NADPH. Mn(III)TMPyP, per se, caused a very slow oxidation of NADPH and this was dramatically increased by cell extract (trace 2). Since 25 μM Mn(III)TMPyP facilitated the complete oxidation of 100 μM NADPH by cell extract without any perceptible stable change in the spectrum of the complex; it follows that the complex acted catalytically, being reduced by the NADPH-dependent oxido-reductase and then reoxidized by dioxygen.

Thioredoxin reductase was able to substitute for cell extract in these measurements. Trace 1 in Figure 8 shows that paraquat strongly stimulated the oxidation of NADPH by thioredoxin reductase, while trace 2 demonstrates that MnTMPyP was also active while the porphyrin without the manganese was not

significantly active (trace 3). Under anaerobic conditions, the MnTMPyP was converted to the green reduced form by thioredoxin reductase plus NADPH. Several other complexes were examined. MnTPyP caused a slow oxidation of NADPH and this was markedly increased by thioredoxin reductase. In contrast, Co(II)TMPyP and Fe(III)TMAP rapidly and non-enzymically oxidized NADPH.

Effect of LB Medium - The observation that LB medium elminiated both the protection by, and the greening effect of, MnTMPyP: suggested that some component of this complex medium was intefering with reduction of this complex. Traces 1 and 2 in Figure 9 show that 20% LB in the reaction mixture inhibited the oxidation of NADPH by cell extract plus MnTMPyP; whereas it had no effect when paraquat was used in place of the Mn-porphyrin (traces 3 and 4). It seems likely that some component of the LB medium, very likely some component of the yeast extract, converts the Mn(III)TMPyP to a form which is not easily reduced by NADPH:Mn(III)TMPyP oxidoreductases, such as thioredoxin reductase.

Non-Enzymic Reduction of Mn(III)TMPyP - The red Mn(III) porphyrin was converted to the green form by incubation with 2-10 mM GSH in phosphate at pH 7.3 or in Tris at pH 7.8. If the green form is indeed the autoxidizable Mn(II)TMPyP then Mn(III)TMPyP should catalyze the oxidation of GSH. Trace 1 in Figure 10 shows that addition of 25 μM Mn(III)TMPyP to 10 mM GSH in

phosphate at pH 7.3 caused an oxygen consumption the rate of which decreased during several minutes of observation. Subsequent addition of additional Mn(III)TMPyP reinstated the more rapid O_2 consumption. Trace 2 records similar effects in Tris at pH 7.8. It appears that Mn(III)TMPyP does catalyze the oxidation of GSH but that it is gradually inactivated during incubation with GSH. The effect of anaerobic incubation with GSH upon the long wavelength absorption spectrum of MnTMPyP is shown in Figure 11. This change in spectrum was also caused by anaerobic incubation of the compound with dithionite, or with NADPH in the presence of thioredoxin reductase, or cell extract.

* * *

All documents cited above are hereby incorporated in their entirety by reference.

One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. One skilled in the art will appreciate from a reading of this disclosure that Mn-porphyrins are not the only compounds that function as oxidoreductases. Riley & Weiss have described a Mn(II)penta-azacyclopentadecane that catalyzes the dismutation of O_2^- with a rate constant 4×10^7 $M^{-1}s^{-1}$ (Riley et al, J. Am. Chem. Soc. 116:387 (1994)). Since the Mn(III) form of this compound is a potent oxidant,

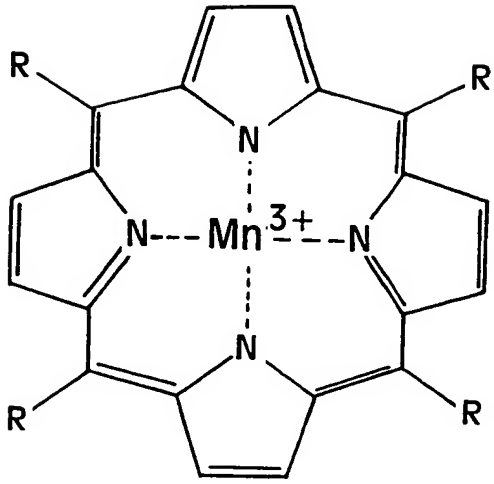
in vivo reaction is likely due to NADPH and GSH, rather than to O_2^-.

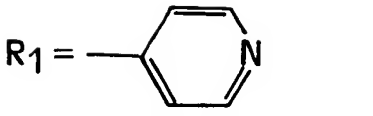
WHAT IS CLAIMED IS:

1. A method of effecting oxidoreduction in a sample containing O_2^- comprising contacting said sample with a metallic porphyrin complex, having substituents on the methine carbons of the porphyrin, under conditions such that said complex is reduced by a reductant and reoxidized by said O_2^-.

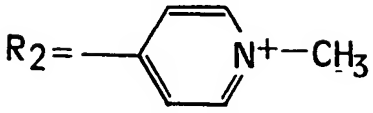
2. The method according to claim 1 wherein said complex is Mn(III) 5,10,15,20-tetrakis(1-methyl-4-pyridyl)-21H,23H-porphine tetra-p-tosylate.

FIG. 1

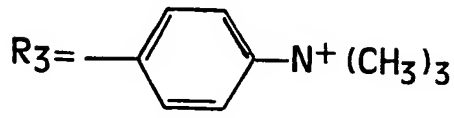




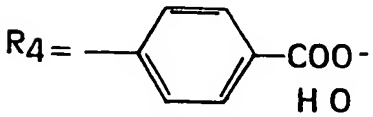
Mn(III) tetra(4-pyridyl)porphyrin
MnTPyP



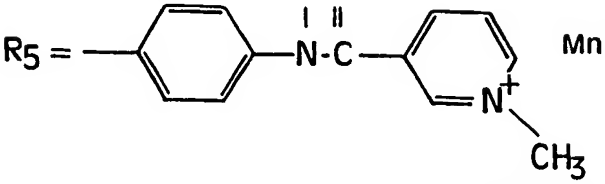
Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin
MnTMPyP



Mn(III) tetrakis(trimethylammonio)phenyl porphyrin
MnTMAP



Mn(III) tetrakis(4-benzoic acid)porphyrin
MnTBAP



Mn(III)α,α,α,β–tetrakis(1-methylisonicotinamido) phenyl]porphyrin
MnTMINP

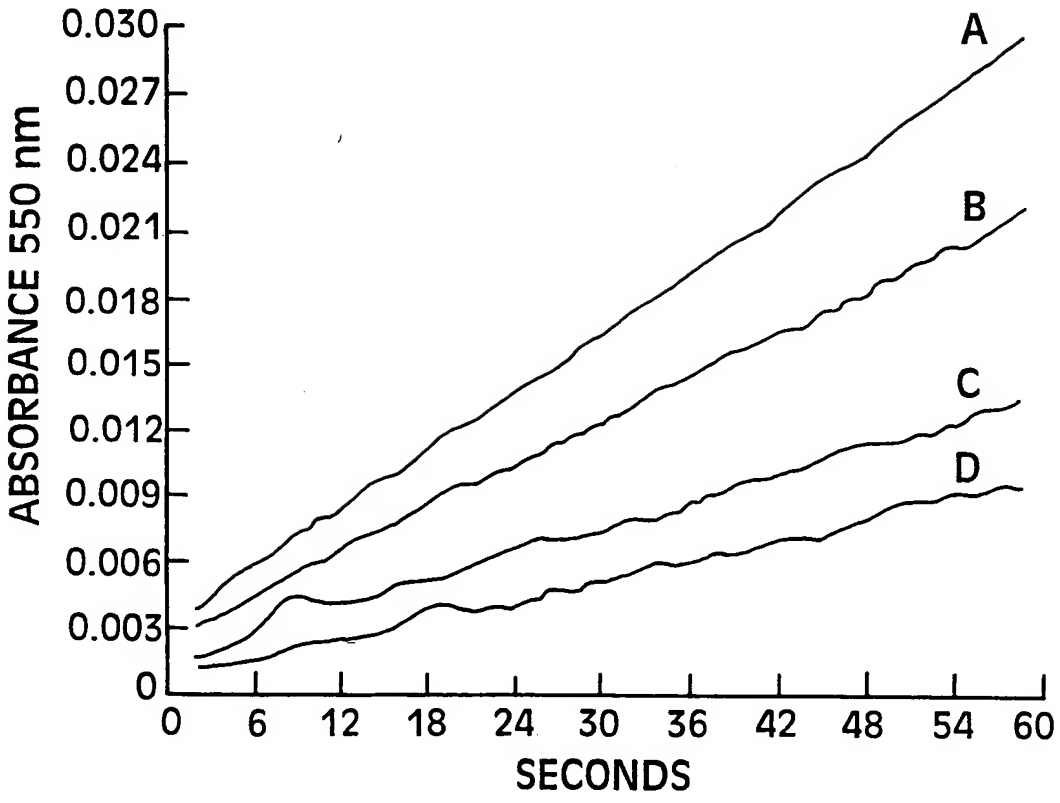
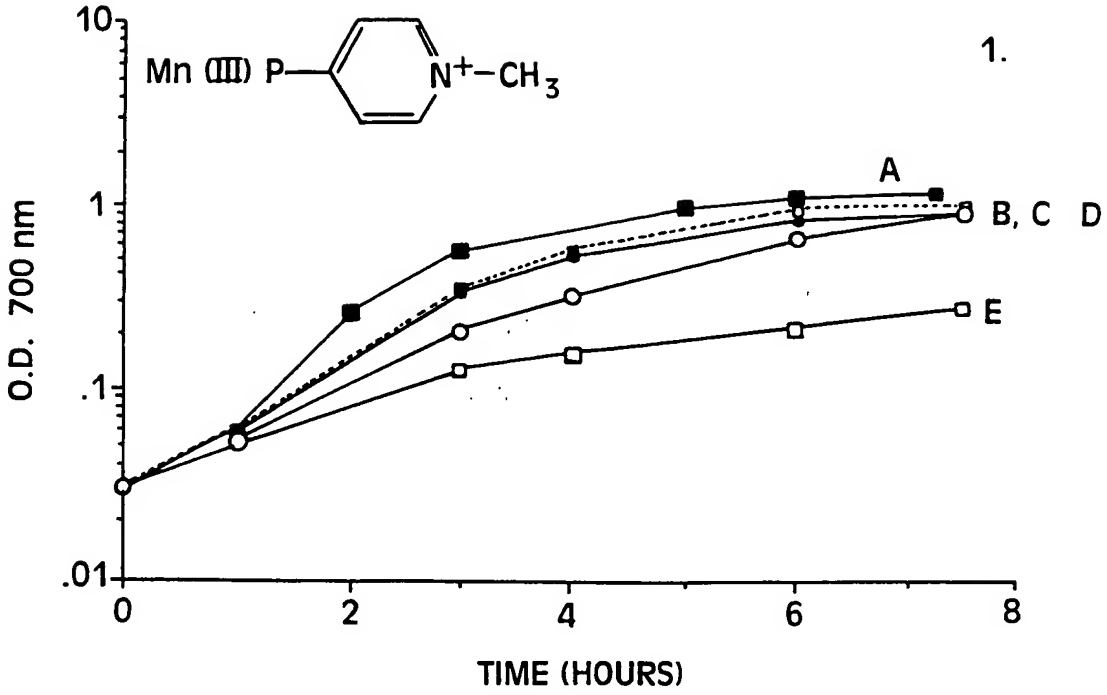

ABSORBANCE 550 nm
0.030
0.027
0.024
0.021
0.018
0.015
0.012
0.009
0.006
0.003
0
0 6 12 18 24 30 36 42 48 54 60
SECONDS
A
B
C
D

FIG. 2


1.
Mn (III) P
N^+-CH_3
A
B, C D
E
O.D. 700 nm
10
1
.1
.01
0
2
4
6
8
TIME (HOURS)

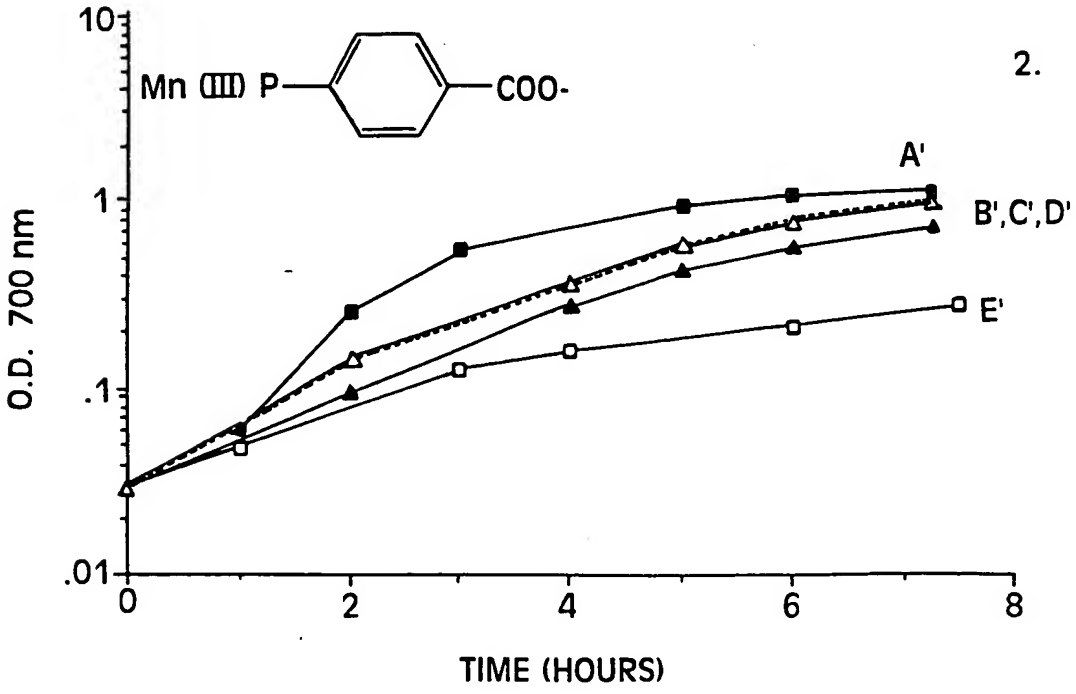

2.
Mn (III) P
COO-
A'
B',C',D'
E'
O.D. 700 nm
10
1
.1
.01
0
2
4
6
8
TIME (HOURS)

FIG. 4

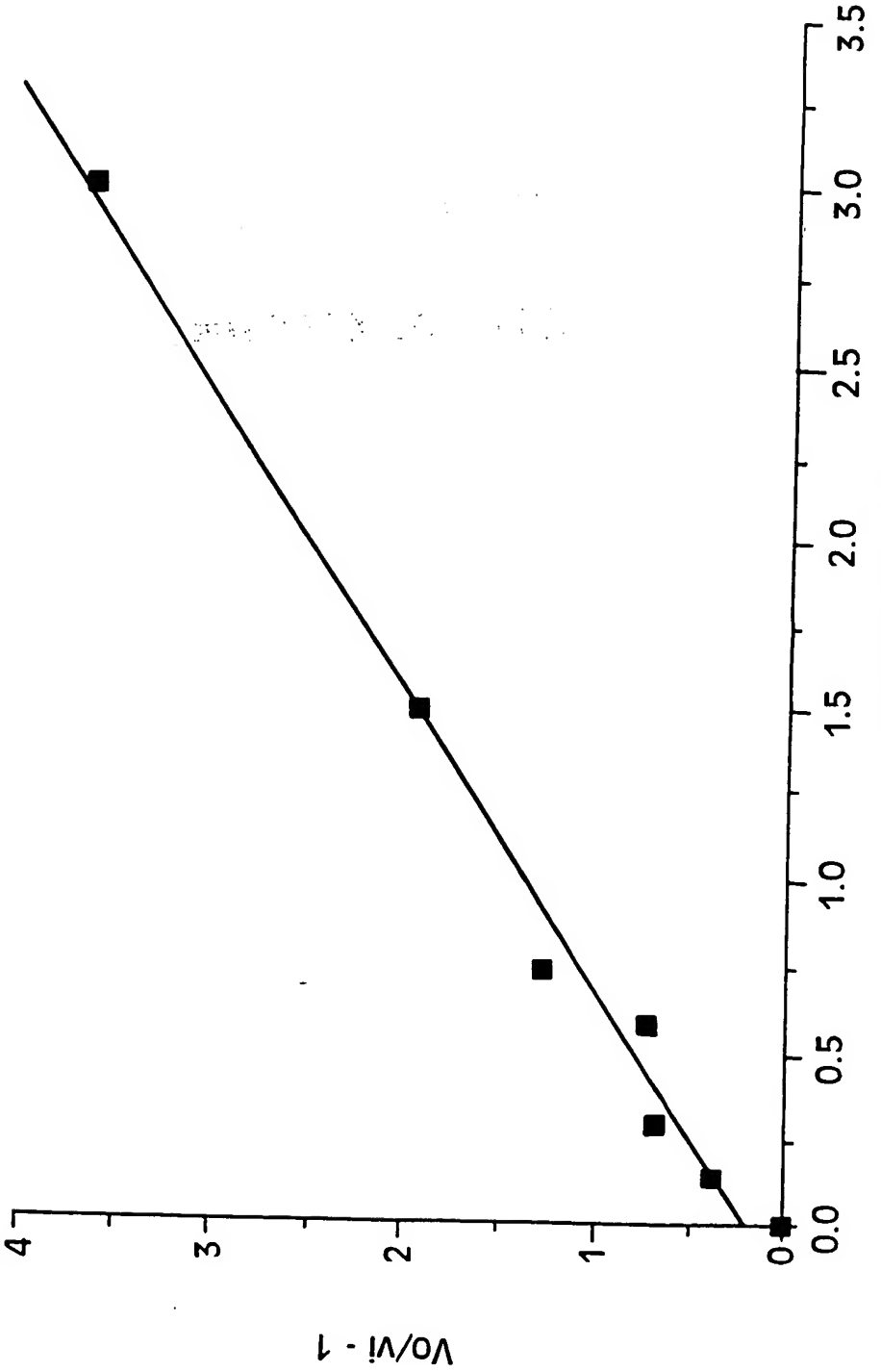


FIG. 3

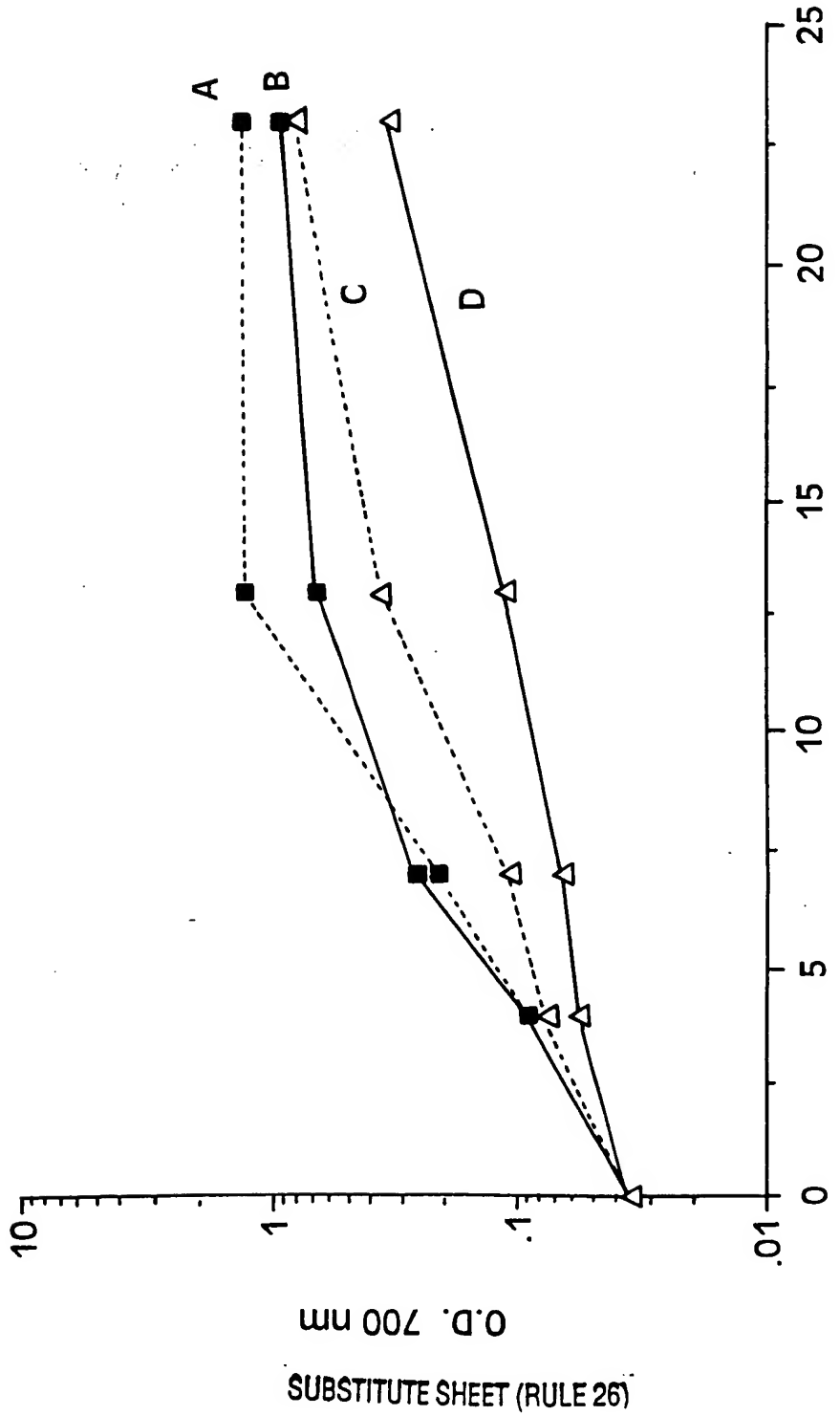
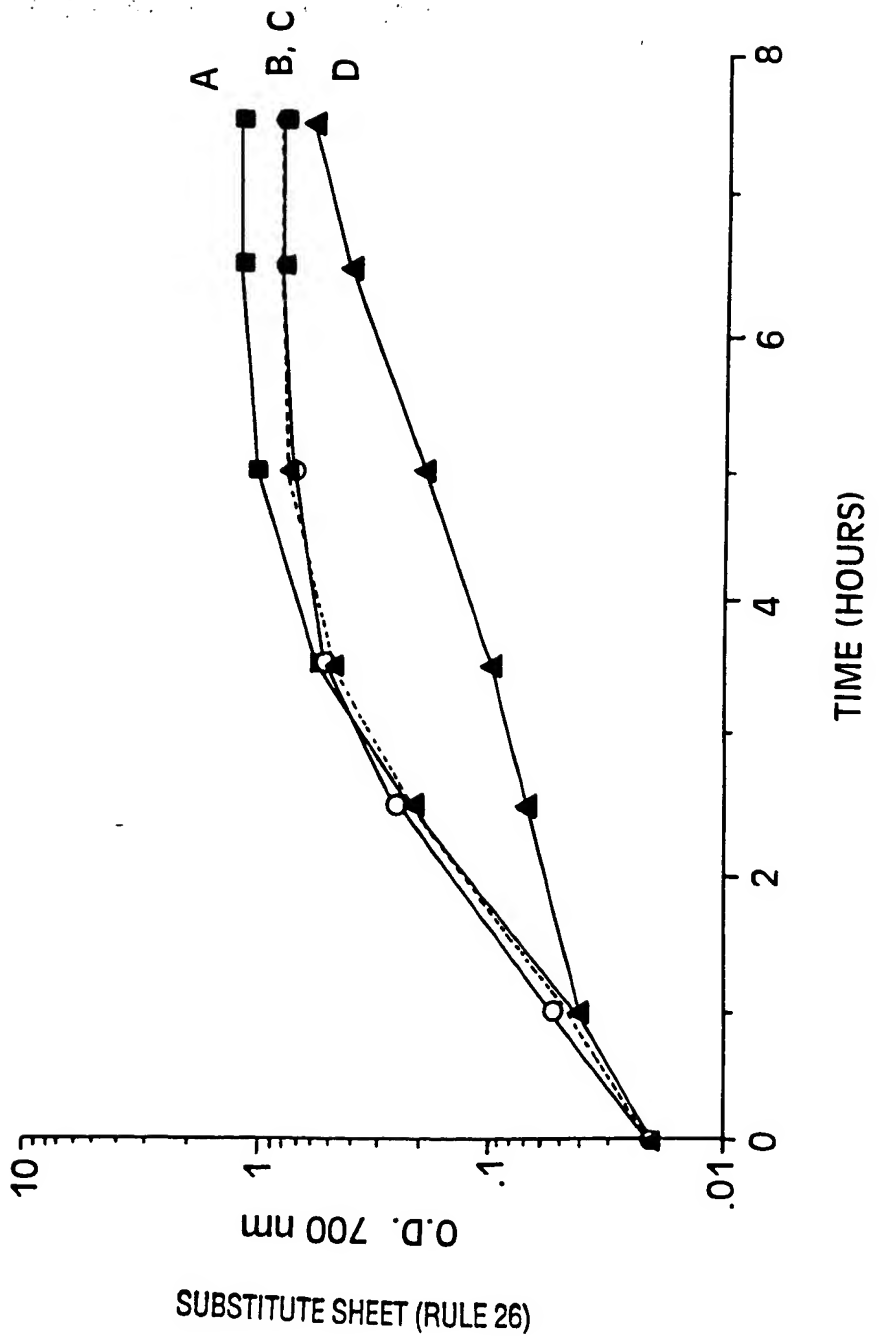


FIG. 5

FIG. 6



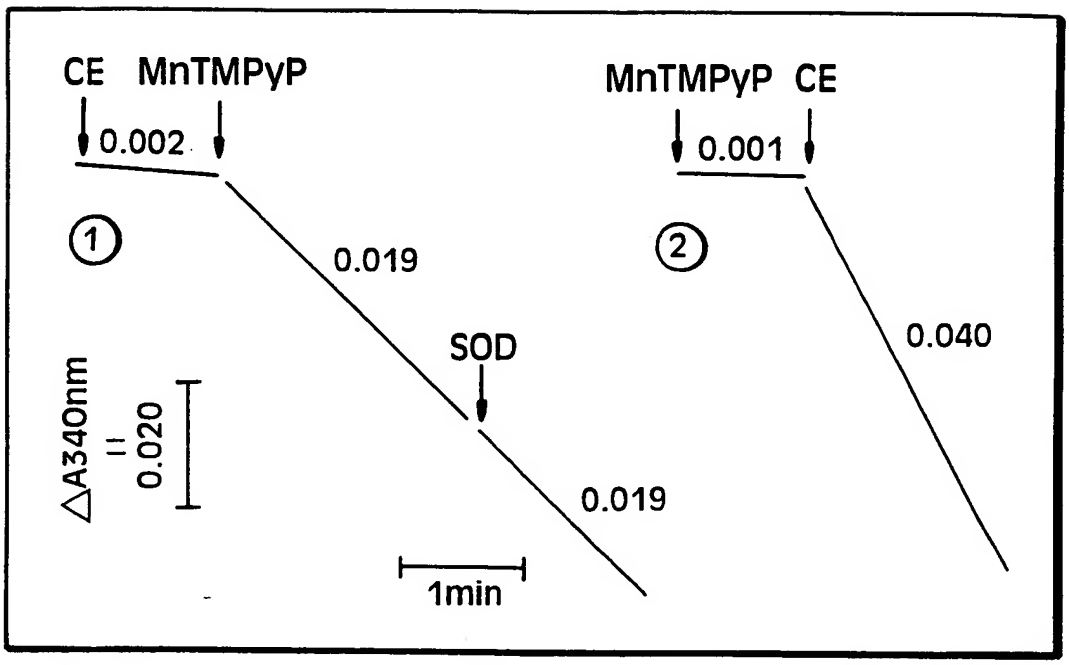

CE
MnTMPyP
0.002
①
0.019
SOD
0.019
1min
ΔA340nm
0.020
MnTMPyP
CE
0.001
②
0.040

FIG. 7

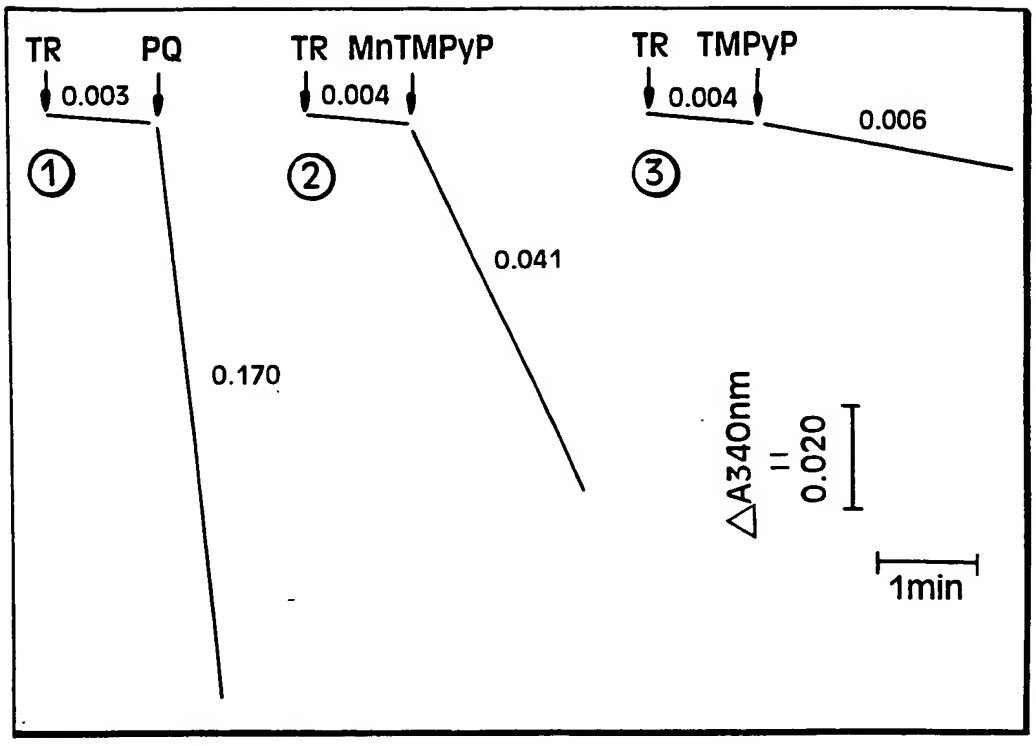

TR
PQ
0.003
0.170
TR MnTMPyP
0.004
0.041
TR TMPyP
0.004
0.006
1
2
3
ΔA340nm
=
0.020
1min

FIG. 8

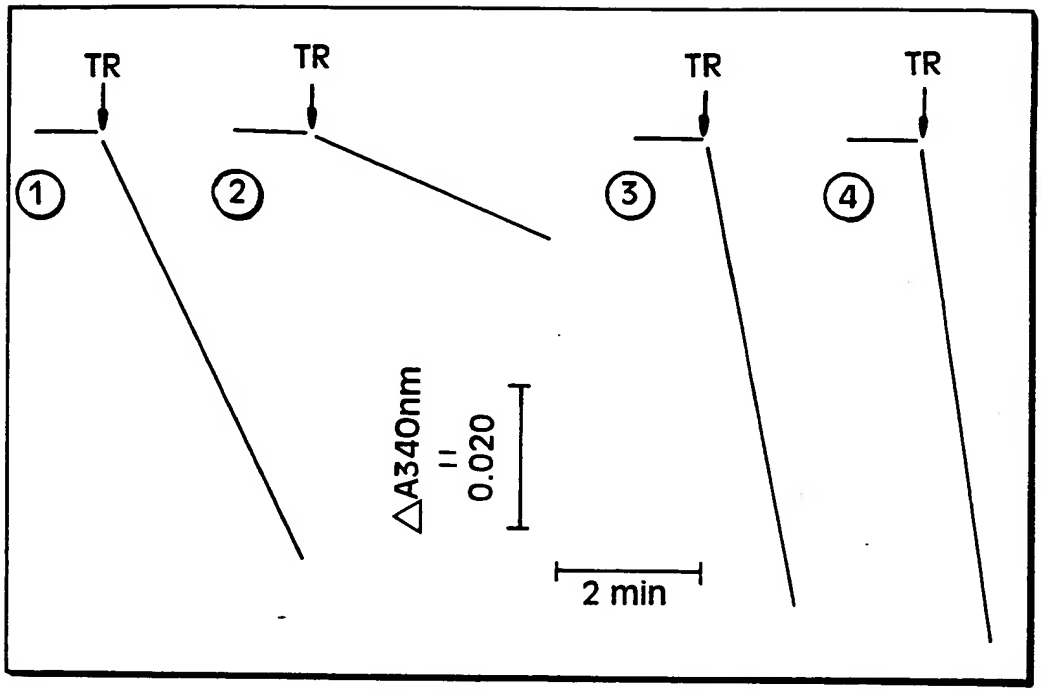


FIG. 9

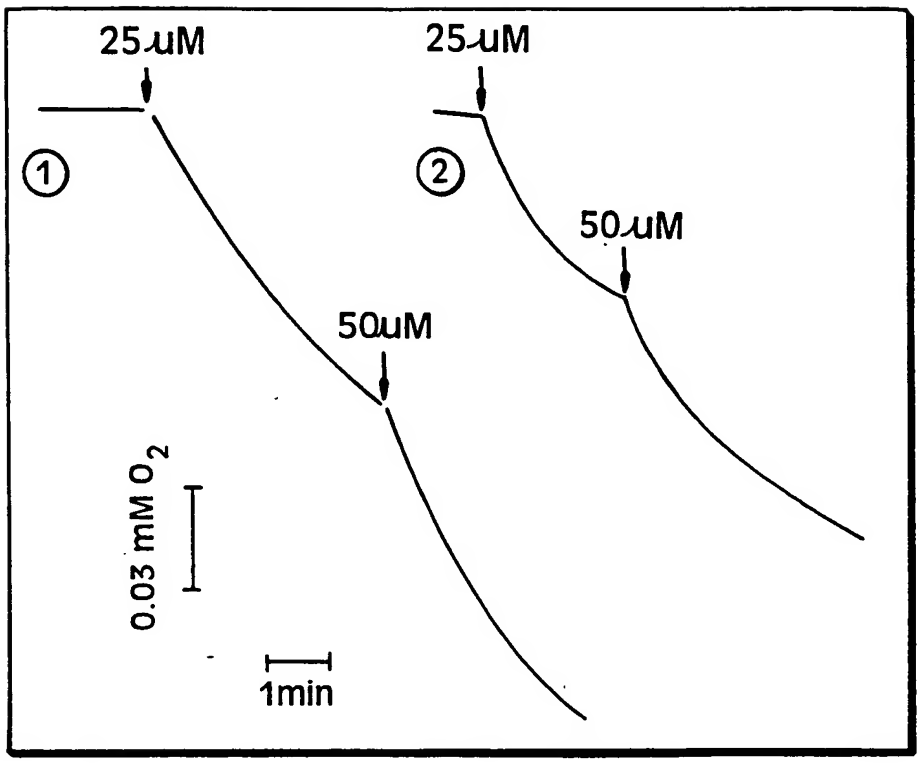


FIG. 10

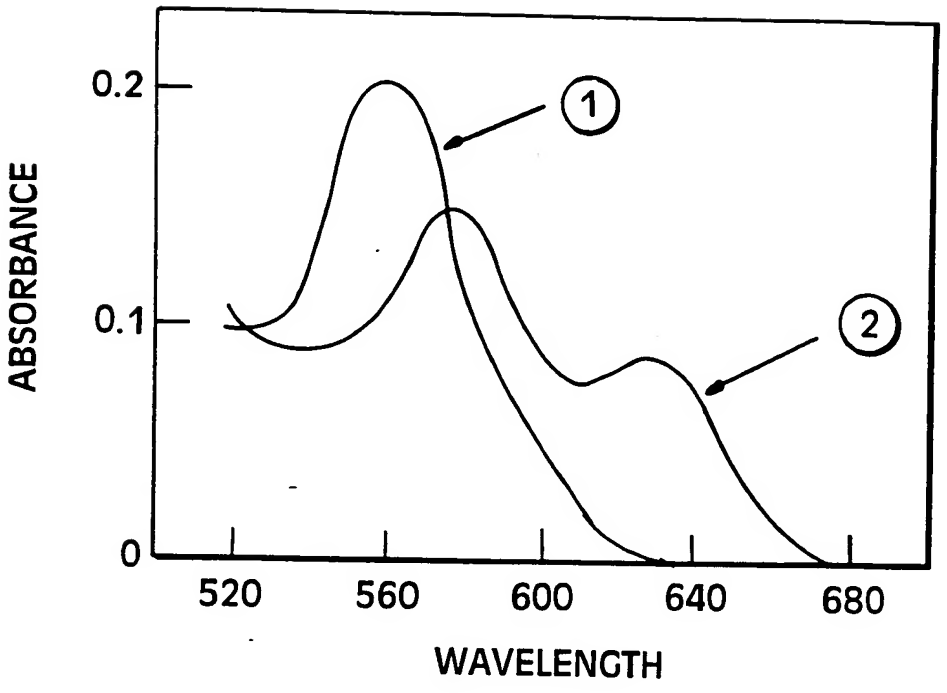


FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/12408

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/555
US CL :514/185

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/185

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,217,966 (BRUICE) 08 June 1993, see claims 16-19.	1-2

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents: -	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 DECEMBER 1995	Date of mailing of the international search report 22 JAN 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KEITH MACMILLAN Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*